PI 3-kinase puts GTP on the Rac

Phosphoinositide 3-kinase, an enzyme that is known to transduce signals received by a variety of receptor types, has been found to mediate agonist-dependent membrane ruffling via the small GTP-binding protein Rac.

It is invariably satisfying to see connections made between hitherto unlinked components of signal transduction pathways. This is particularly true when the two components carry with them a large literary baggage; the consequence is often that a lot of previously purely descriptive results begin to make mechanistic sense. In a paper that appeared recently in *Current Biology* [1], Hawkins and colleagues have drawn together two such disparate strands to provide the basis for a molecular description of the mechanisms responsible for the agonist control of a well-described eukaryotic cellular phenomenon — membrane ruffling.

The characters involved in this plot are a lipid kinase — phosphoinositide 3-kinase (PI 3-kinase) — and the small GTP-binding protein Rac. Why is there such interest in PI 3-kinase? As its name implies, PI 3-kinase catalyzes the phosphorylation of the inositol ring of its lipid substrate at the 3-OH position (see Fig. 1). Thus, in contrast to the classical pathway of signal-dependent inositol lipid hydrolysis — in which activated phospholipase C releases the two signalling molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) — PI 3-kinase generates the highly phosphorylated lipid, phosphoinositide 3,4,5-trisphosphate (PIP_3). PI 3-kinase can also generate PIP and PIP_2 — from substrates PI and PIP, respectively — but labelling studies [2] suggest that PIP_3 is the important signal-generated product.

PI 3-kinase was originally identified in association with particular activated receptor tyrosine kinase complexes [3,4], and it has subsequently been found in many others (reviewed in [5]). The enzyme was found to consist of two subunits: an 85 kD regulatory subunit, with two 'Src homology 2' (SH2) domains, and a 110 kD catalytic subunit [6] which shows homology to a yeast vacuolar protein sorting gene product (VP34p, which does indeed possess PI 3-kinase activity [7]). The two SH2 domains of the 85 kD regulatory subunit enable it to bind tyrosine-phosphorylated sequences within the cytosolic domains of receptor tyrosine kinases, explaining how PI 3-kinase becomes assembled on activated, autophosphorylated, receptor tyrosine kinase complexes.

The activation of p85/p110 PI 3-kinase that is consequent on ligand stimulation of a receptor tyrosine kinase is likely to be due to a combination of factors. These are, first, binding of the enzyme to the tyrosine phosphorylated target motif (the peptide YMXM, in the single-letter amino-acid code); second, the fact that complex assembly occurs at the membrane, thus bringing the enzyme in close proximity with its lipid substrate; and third, direct interaction of the enzyme with the small GTP-binding protein Ras [8,9]. Activation of PI 3-kinase leads to production of PIP_3, but how is the signal transmitted downstream of this lipid metabolite?

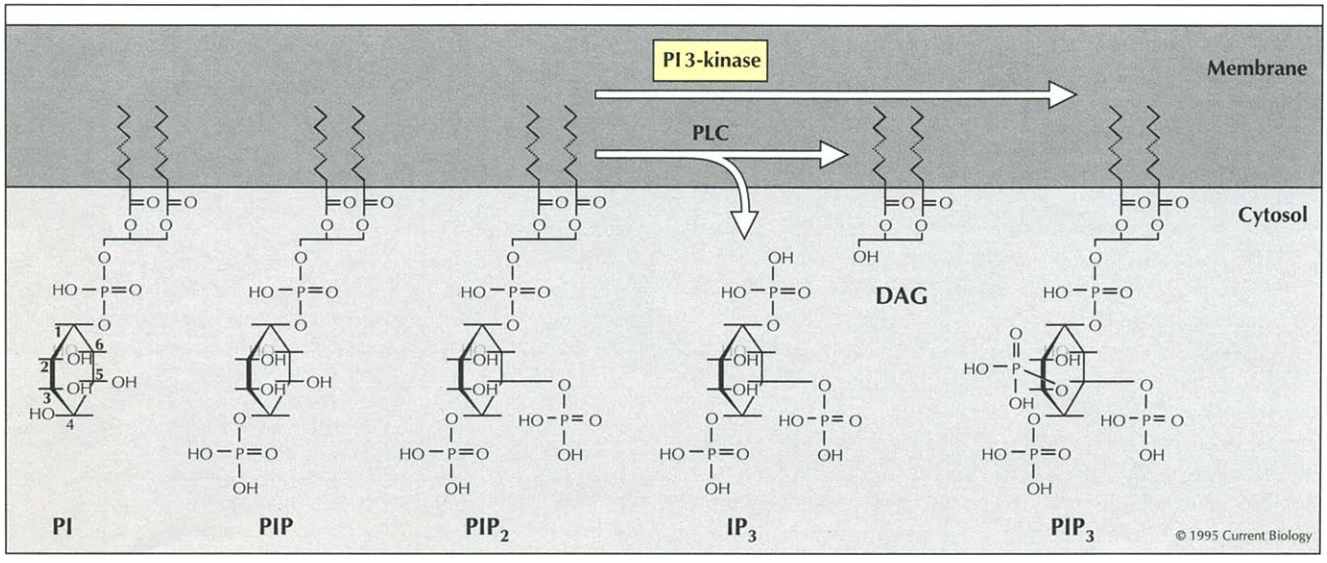

Fig. 1. Two pathways from phosphatidylinositol are important in the generation of intracellular signalling molecules. The pathways diverge at PIP_2: PI 3-kinase catalyzes phosphorylation of this lipid, generating PIP_3; phospholipase C (PLC) catalyzes hydrolysis of PIP_2, generating IP_3, which activates release of Ca^{2+} from internal stores, and diacylglycerol (DAG), which activates protein kinase C.

The recent advances in understanding the role of PI 3-kinase as an upstream element in an important intracellular signalling pathway have in large part been made possible by the availability of a specific inhibitor of the enzyme, the anti-fungal agent wortmannin. This, and certain related compounds, have been shown to inhibit PI 3-kinase with great potency (K_i ~ 10 nM), both *in vitro* and *in vivo* [10,11]. At higher concentrations of wortmannin, other proteins are also affected, but to date PI 3-kinase is the only known high-affinity target in mammalian cells. Wortmannin inhibits a number of cellular responses, in particular the activation of neutrophils, suggesting that PI 3-kinase is a component of the intracellular signalling pathways that mediate these responses.

Of particular relevance for the recent work of Hawkins *et al.* [1] — discussed in more detail below — wortmannin has been shown to inhibit the membrane ruffling response of cells that is observed following stimulation with certain agonists [12,13]. Indeed, in the course of their recent study, Hawkins *et al.* [1] have shown that, in porcine aortic endothelial cells, the ruffling response to platelet-derived growth factor (PDGF) is blocked not only by wortmannin, but also by a mutant form of the p85 subunit of PI 3-kinase that does not bind to the p110 catalytic subunit to direct its activation. This observation brings us to the second character in the story, the small GTP-binding protein Rac.

The family of small GTP-binding proteins encompasses four classes, namely the Ras, Rho (which includes Rac), Rab and ARF (ADP-ribosylation factor) families. Each class, broadly speaking, appears to be involved in a particular area of cell biology. Thus, Ras family proteins can regulate various growth responses [14], Rab family proteins regulate vesicle sorting pathways [15], and ARF, which is also involved in vesicle traffic, has recently been implicated in the control of phospholipase D [16]. In each of these contexts, the loading of the GTP-binding protein with GTP catalyzed by a GTP–GDP exchange protein is the critical first step in its cyclical action.

The active, GTP-bound protein subsequently interacts with its target 'effector' proteins, the activities of which are modified as a result of the interaction. The GTP-bound protein reverts to the inactive state on GTP hydrolysis, promoted either by a specific GTPase-activating protein (GAP) or by the interaction with the target protein itself. So where does the Rho family fit in? Seminal work from Ridley, Hall and colleagues (see [17,18]) has shown that members of this family — Cdc42, Rho and Rac — are regulators of the actin cytoskeleton. For example, microinjection of GTP-bound Rho has been shown to induce actin stress fibres in fibroblasts. Similarly, microinjection of active Rac will induce a reorganization of the membrane-associated actin cytoskeleton — the cortical cytoskeleton — producing a characteristic membrane ruffling response.

So, PI 3-kinase and Rac are connected by their involvement in the control of membrane ruffling. Hawkins *et. al.* [1] have now provided evidence that these two elements operate in a hierarchy, wherein activated PI 3-kinase increases the level of the GTP-bound, active form of Rac, which in turn induces the changes in the actin cytoskeleton responsible for membrane ruffling (Fig. 2). The first line of evidence for this comes from the experiments with inhibitors alluded to above. Thus, they found that treatments that inhibit PI 3-kinase — wortmannin and the dominant-negative mutant form of p85 — block membrane ruffling that is normally induced in an endothelial cell line by PDGF; these treatments did not, however, block membrane ruffling induced directly by injection of a constitutively active mutant form of Rac.

The second line of evidence comes from direct measurements of the level of GTP loading on Rac *in vivo*. The ratio of GDP:GTP bound forms of Rac is determined by the rate of nucleotide (GTP for GDP) exchange and the rate of hydrolysis of Rac-bound GTP. Unlike the situation with Ras, with Rac it had previously been impossible to determine the ratio of bound GDP:GTP *in vivo* (the requisite antibodies not being available). To circumvent this obstacle, stable cell lines were established expressing an epitope-tagged form of Rac that permits immunoisolation from cells (control or stimulated) and determination of the GDP:GTP ratio. PDGF was found to increase the GTP–Rac level 1.7-fold, and this effect is blocked by the PI3-kinase inhibitor wortmannin. Interestingly, this increase was found to result from the combination of a

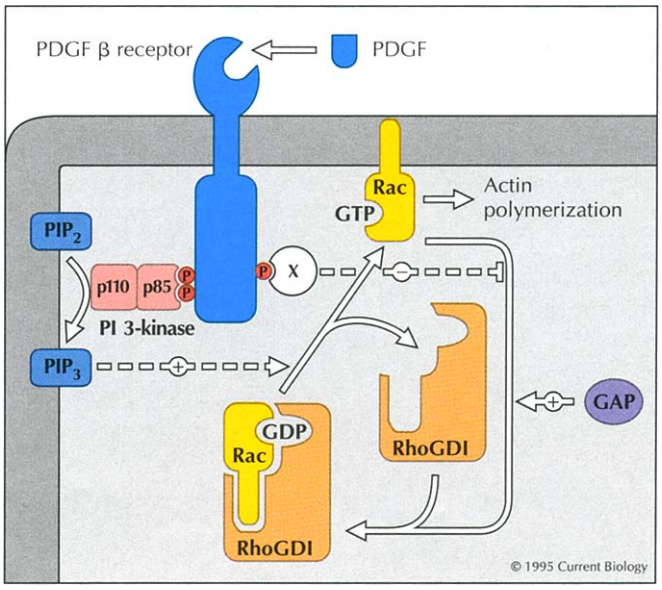

Fig. 2. PDGF receptor coupling to actin polymerization. The activated, tyrosine-autophosphorylated PDGF receptor is shown interacting with the heterodimeric PI 3-kinase (p85/p110). The consequent activation leads to PIP_3 production, which is in turn responsible for causing GDP–Rac to dissociate from its binding protein GDI (for 'guanine nucleotide dissociation inhibitor') and/or exchange GDP for GTP, releasing GTP–Rac, which somehow stimulates actin polymerization. A second PDGF-receptor-dependent, PI3-kinase-independent, pathway, mediated by a putative transducing component 'X', amplifies the response by inhibiting Rac GTPase activity (which is itself stimulated by GAP).

wortmannin-sensitive increase in GTP–GDP exchange on Rac, and a wortmannin-insensitive decrease in Rac GTPase activity (Fig. 2). The central conclusion, then, is that PDGF, by activating the PI 3-kinase, causes an increase in the level of GTP–Rac, which in turn controls actin polymerization and hence membrane ruffling.

Is Rac activation the sole result of PI 3-kinase action? The ability of wortmannin to block other responses suggests not. For example, wortmannin has been shown to block up-regulation of the glucose transporter GLUT4 in pancreatic islet cells in response to insulin [19]. Although PDGF and insulin can both induce a robust activation of PI 3-kinase, only insulin can induce GLUT4 up-regulation. This distinction appears to reflect the differential localization of the activated PI 3-kinase (P. Shepherd and K. Siddle, personal communication) and the consequent compartmentalized nature of the lipid product, PIP_3. Unlike second messengers such as cAMP, which are free to diffuse as water-soluble entities, lipid messengers are largely restricted to the membrane compartments in which they are made, at least until that compartment buds from, or fuses with, another. Thus, activation of PI 3-kinases in distinct locations may have profoundly different consequences. A further potential source of diversity is the existence of multiple, distinct PI 3-kinases, though whether this is relevant to the selective responsiveness of islet cells is not known.

Another pathway, involving the activation of the $p70^{S6kinase}$, does, however, appear to involve a distinct, but related, PI 3-kinase. This signalling event is sensitive to the immunosupressive drug rapamycin, which has been shown [20] to interact in the yeast *Saccharomyces cerevisiae* with two gene products, TOR1 and TOR2, that encode PI 3-kinase-related proteins. Rapamycin does not inhibit the p85/p110 PI 3-kinase, but in mammalian cells it does inhibit $p70^{S6kinase}$ activation [21], implying the existence of a distinct agonist-regulated PI 3-kinase signalling pathway. At least two further PI 3-kinase activities have been identified, one regulated by seven-transmembrane-helix receptors via heterotrimeric G protein $\beta\gamma$ subunits [22], and one that shows a specificity for the lipid PI — as opposed to PIP_2 — as substrate [23]. This suggests that the compartmentalization afforded by the lipid products of these distinct activities may be involved in a variety of cellular control processes.

Although the plethora of events known to involve PI 3-kinases is still growing, no others have been connected in the manner described by Hawkins *et al.* [1] for Rac activation. Inevitably, then, this system will be fertile ground for further dissection. How the increase in GTP–GDP exchange on Rac is effected, and the means by which GTP–Rac controls local actin polymerization are evidently crucial outstanding issues. Perhaps the most intriguing question, however, is whether the results of Hawkins *et al.* have revealed a general principle of PI 3-kinase action — are all the responses to PI 3-kinase mediated via GTP-binding proteins?

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